IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Darji et al.

Application No.	09/419,545
Filed:	October 18, 1999
For:	ATTENUATED SALMONELLA STRAIN USED AS A VEHICLE FOR ORAL IMMUNIZATION

DECLARATION

1. I, Dr. Siegfried Weiß, residing in Mascheroder Weg 1, 38124 Braunschweig, Germany am a citizen of the Federal Republic of Germany. I am holding a PhD in Molecular Biology of the Free University of Berlin. I am head of the research group Molecular Immunology, Division of Molecular Bitechnology at the Gesellschaft für Biotechnologische Forschung, German Research Centre for Biotechnology, Mascheroder Weg 1, 38124 Braunschweig, Germany since 1990. I enclose my Curriculum vitae.

I am an inventor of the US patent application filed on October 18, 1999 under the Application No. 09/419,545 for: "Attenuated *Salmonella Strain* used as a vehicle for oral immunization".

I am trained and skilled in the field development of immunization vectors and vehicles, in particular *Salmonella*. I have done extensive research in the field bacterial research. I have conducted numerous experiments myself and supervised MD and PhD students in doing so. A list of publications in renowned scientific journals is enclosed.

2. I am entirely aware of the disclosure of our US patent application Serial No. 09/419,545 filed on October 18, 1999.
Additionally, I have studied and fully understood the USPTO Office Action dated 09/28/2005 and the prior art references to our US patent application:

i) US patent 5,877,159 (Powell et al.)
ii) US patent 5,824,538 (Branstrom et al.), and
iii) Sizemore et al. (1995), Science, Vol. 270, p. 299-302

3. The invention described in our US patent application 09/419,545 concerns an attenuated strain of *Salmonella*, in particular *Salmonella typhimurium*, which has been used as a vehicle for oral genetic immunization. Eukaryotic expression vectors containing truncated genes of ActA and listeriolysin, which are two virulence factors of *Listeria monocytogenes*, have been used to transform *Salmonella typhimurium*. Multiple and even single oral immunizations with such transformants induced excellent cellular and humoral immune responses. We were able to also induce protective immunity. The quality of the immune response was unexpectedly good. We presumed that a transfer of plasmid DNA from the bacterial carrier to the host takes place, and we were able to unequivocally prove this estimation by showing the transfer with primary peritoneal macrophages *in vitro*.

4. The Examiner argues that Powell et al. were the first researchers to have shown that bacteria can be used as vehicles for the transfer of eukaryotic expression plasmids into animal cells. This is incorrect. W.

Schaffner (Proc Natl Acad Sci U S A. 1980 Apr;77(4):2163-7) had published this already in 1980. The mechanism was most likely a conjugational transfer. The frequency of transfer was, however, impractical for general use especially *in vivo*.

5. The prior art reference (i) US patent 5,877,159 (Powell et al.) deals with a method for introducing and expressing genes in animal cells, which comprises infecting the animal cells with live invasive bacteria, wherein the bacteria contain a eukaryotic expression cassette encoding said gene (see Abstract of Powell et al.). Powell and co-workers assert that "The particular Salmonella strain employed is not critical to the present invention. Examples of Salmonella strains which can be employed in the present invention include *Salmonella typhi* (ATCC No. 7251) and *S. typhimurium* (ATCC No. 13311). Attenuated Salmonella strains are preferably used in the present invention and include *S. typhi* aroAaroD (Hone et al, *Vacc.*, 9:810-816 (1991)) and *S. typhimurium* aroA mutant (Mastroeni et al, *Micro. Pathol.*, 13:477-491 (1992)))." (c.f. column 13, lines 14 to 22). However, the experimental section of the '159 patent was only carried out with an attenuated *Shigella flexneri* strain (c.f. column 20, line 29; column 22, lines 46 to 47 and in particular examples 5 to 7 in columns 25 to 26.)

6. The Examiner argues that Powell et al. have shown the principle of DNA transfer from bacteria to animal cells at reasonable frequencies by using ***Shigella flexneri*** as transfer vehicle, therefore it was clear that many bacteria including *Salmonella typhimurium* and *Salmonella typhi* should be able to do the same. However, the contrary is the case. In their publication Sizemore et

al. (Science. 1995 Oct 13;270(5234):299-302) state clearly that it is not possible to use *Salmonella typhimurium* as transfer vehicle because the bacteria do not escape from the phagocytic vacuole while *Shigella flexneri* do. The same statement is found in their patent which was approved on Oct. 20, 1998, US 5,824,538 (cf. e.g. columns 19 and 20, bridging paragraph). Thus, the statement that *Salmonella* can deliver DNA vaccines had no basis in the art at that time. Actually, at that time it was believed that such bacteria need to be equipped with lysins that allow the escape of the bacteria from the phagocytic vacuole (Courvalin et al.: C R Acad Sci III. 1995 Dec;318(12):1207-12).

7. Additionally, with the experiments carried out by Powell and co-workers, they would not have been able to show the transfer of eukaryotic plasmids. They only used established cell lines, in which no transfer can be observed. In our patent application, we are the first to show that also *Salmonella* species are able to transfer eukaryotic expression plasmids. However, it worked only and with unforeseeable high frequencies when particular animal cells were used, namely isolated macrophages, especially freshly isolated macrophages; c.f. US 2003/0180320 A1, for example [0034] and [0048]. With established cell lines as had been used in the competing applications no transfer is observed. Therefore, in the art the possibility to use *Salmonella* species for transfer of DNA vaccines had been depreciated before our publication and patent application.

8. I further declare, that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and

further that these statements are made with the knowledge that willful false statements and the like, so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the US Code, and that such willful false statements my jeopardize the validity of the application or any patent issuing thereon.

March 24, 2006 [signature]

Date and Signature
[Siegfried Weiß]

March 24, 2006 [signature]

Date and Signature witness

[Sara Leschner]

Curriculum vitae - Siegfried Weiss

Born:	28th February, 1949
In:	Fürth, Germany
1955-1959:	Primary School
1959-1969:	Grammar School (Gymnasium)
1969-1970:	Education Department of the Faculty of Philosophy at the University of Erlangen-Nürnberg.
1969-1978:	Biology Department of The Free University of Berlin Special Studies: Molecular Biology and Genetics.
1973-1974:	Thesis: "Zur Struktur der DNA von SPP1" (On the structure of the DNA of SPP1), Max-Planck-Institute for Molecular Genetics, Berlin, Germany; Supervisor: Dr. T. A. Trautner.
1974-1978:	PhD thesis: "Origin and Immunochemical Characteristics of the Variant Rabbit strain BASILEA", Basel Institute for Immunology, Basel, Switzerland; Supervisor: Dr. A. S. Kelus.
1978:	PhD (Dr. rer. nat.) graduated at the Free University of Berlin
1978-1980:	Research Associate with a research fellowship of the Deutsche Forschungsgemeinschaft (German Research Council) at the Salk Institute for Biological Studies, Department M. Cohn, San Diego, USA.
1980-1983:	Scientific Assistant at the Friedrich-Miescher-Laboratory of the Max-Planck-Gesellschaft, Tübingen, Germany.
1983-1989:	Member of the Basel Institute for Immunology, Basel, Switzerland.
1989-1990:	Visiting Professor at the Institute for Immunology and Rheumatology, University of Oslo, Oslo, Norway.
1990-present:	Group leader at the GBF (Gesellschaft für Biotechnologische Forschung), Braunschweig, Germany

Braunschweig, March 2006

Publikationen - Siegfried Weiss

1. A. S. Kelus, S. Weiss, 1977:
   Variant strain of rabbits lacking immunoglobulin κ polypeptide chain. *Nature* 265, 156-158.

2. S. Weiss, A. S. Kelus, D. G. Braun, 1977:
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4. S. Weiss, G. Dennert, 1981:
   T cell lines active in the delayed-type hypersensitivity reaction (DTH). *J. Immunol.* 126, 2031-2035.

5. G. Dennert, S. Weiss, J. F. Warner, 1981:
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   The ratio of δ- to δ-bearing cells in murine fetal liver and bone marrow. *The Immune System* 2,33-37.

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8. S. Weiss, K. Lehmann, M. Cohn, 1983:
   Monoclonal antibodies to murine immunoglobulin isotypes. *Hybriodoma* 2, 49-54.

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   A Mott cell hybridoma. *Eur. J. Immunol.* 14, 744-748.

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    V82 rearranges with all functional J8 segments in the mouse. *Eur. J. Immunol.* 15, 765-768.

12. S. Weiss, K. Lehmann, M. Cohn, 1985:
    A monoclonal δ1 bearing anti-Dextran antibody from a δ defective mouse strain. *Eur. J. Immunol.* 15, 768-772.

13. A. S. Kelus, S. Weiss, 1986:

Publikationen - Siegfried Weiss

1. A. S. Kelus, S. Weiss, 1977:
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mal growth factor, fibroblast growth factor, or angiotensin II all produce a rise in $[H_2O_2]$ with a correlation existing between the magnitude and duration of an increase in $[H_2O_2]$ and the level of tyrosine phosphorylation (*10*). These latter observations also strengthen the case that in many ways H_2O_2 fulfills the definition of an intracellular second messenger.

VSMCs appear to be unusual in their uptake of extracellular catalase. Certain cells secrete catalase, and the amount of catalase in serum increases in certain disease states (*14*). Thus, growth of VSMCs might be influenced by extracellular catalase in vivo. Recent epidemiological studies suggest a cardioprotective effect of antioxidants (*15*). Given that PDGF-induced VSMC migration and proliferation is thought to precipitate early atherogenic changes (*16*), one mechanism by which dietary antioxidants might protect against cardiovascular events is by a direct effect on H_2O_2-mediated signal transduction in VSMCs.

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17. Primary VSMCs were obtained from rat thoracic aorta by enzymatic digestion as described [J. H. Campbell and G. R. Campbell, *Vascular Smooth Muscle in Culture* (CRC, Boca Raton, FL, 1987), pp. 15–22] and were stimulated with PDGF-AB (5 ng/ml); DCF fluorescence was measured (*6*). Images were collected with a Leica Laser confocal scanning microscope, model TCD4. Relative DCF fluorescence was recorded on a scale from 0 to 256. In general, basal fluorescence averaged from 0 to 10 units, whereas stimulated fluorescence averaged from 60 to 200 units. Although DCF is oxidized by both H_2O_2 and hydroxyl radicals, the lack of fluorescence in PDGF-stimulated catalase-loaded cells suggests that the fluorescent signal after the addition of growth factor is predominantly derived from H_2O_2 (see Fig. 2E).
18. VSMCs deprived of serum for 3 days were stimulated with the indicated concentration of H_2O_2 or PDGF-AB (5 ng/ml) for 20 min, after which cells were harvested and lysed in RIPA buffer [50 mM tris-Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS]. Immunoprecipitation was done from 150 mg of unstimulated or stimulated cell lysate with aphosphotyrosine antibody (4G10, UBI). Immunoprecipitated proteins were divided in equal portions, and tyrosine-phosphorylated proteins from 50 mg of lysate were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 20% gels) and transferred to a nitrocellulose filter. When indicated, filters were probed with either an antibody to phosphotyrosine (RC20), a broadly reactive antibody to MAP kinase (erk1-CT, UBI), or an antibody to paxillin (P13520, Transduction Lab). Amounts of MAP kinase protein were detected from total cell lysates (10 μg per lane) by erk1-CT. Antibody binding was visualized by enhanced chemiluminescence (Tropix).
19. Adenoviral stocks were prepared and titered on 293 cells and used to infect VSMCs as described [R. J. Guzman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10732 (1994)]. Three days after infection, cells were deprived of serum for 3 days, then triplicate cultures were collected in buffer A [1× phosphate-buffered saline (PBS) (0.0067 M), 10 mM EDTA, 2% Triton X-100, and 0.5% deoxycholic acid] for measurement of enzymatic activity or in RIPA buffer for analysis with antibodies to phosphotyrosine.
20. Confluent VSMCs (1 x 10^6 cells), HUVECs [American Type Culture Collection (ATCC), Rockville, MD], and HeLa cells (ATCC) that had been deprived of serum were incubated with beef liver catalase (3000 U/ml, Boehringer Mannheim) for various times. After incubation, cells were washed twice in PBS, trypsinized, and lysed in buffer A. Catalase activity was assayed by the rate of decrease in absorbance at 240 nm by the method described by H. Aebi [*Methods Enzymol.* **105**, 121 (1994)].
21. Cells incubated with extracellular catalase (3000 U/ml) were collected, and triplicate cultures were exposed to proteinase K (1 mg/ml) in solution for various times. At the indicated time, cells were quickly sedimented and proteinase K diluted and removed. The cells were subsequently lysed in buffer A. Residual catalase activity was measured as described (*20*).
22. Confluent VSMCs with and without catalase loading were stimulated for 30 min with 10 mM SNP. Cells were subsequently lysed with 6% trichloroacetic acid (TCA). Lysates were neutralized, and equal amounts (2 x 10^6 cells) were used to determine amounts of cGMP by radioimmunoassay (Amersham) according to the manufacturer's recommendation.
23. Cells were stimulated with PDGF (5 ng/ml) for 20 min, and MAP kinase proteins were partially purified by phenylsepharose chromatography as described [S. Offermanns, *J. Immunol.* **152**, 250 (1994)]. Myelin basic protein (MBP) phosphorylation was used as an index of MAP kinase activity, where 100% activity represents the activity derived from PDGF-stimulated cells in the absence of catalase loading.
24. Confluent VSMCs were maintained in media without serum for 3 days and then, as indicated, stimulated with PDGF-AB (5 ng/ml). After 18 hours, cells in quadruplicate wells were incubated for 3 hours with [^{3}H]thymidine (1 mCi/ml), and TCA-precipitable counts were determined. If VSMCs were stimulated with PDGF in the presence of 0.5% serum, we noted effects on both basal and PDGF-stimulated thymidine incorporation; under serum-free conditions, effects were present only with PDGF stimulation. This was presumably because of the effects of catalase loading on residual growth factors present under 0.5% serum conditions.
25. VSMC migration was measured in response to PDGF stimulation in a mini-Boyden chamber as described [S. Biro *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 654 (1993)]. In the absence of PDGF stimulation, there was on average 10 VSMCs per high-power field.
26. A 200-mM stock of NAC was adjusted to pH 7.4 by NaOH, flash frozen, and stored in portions at −80° for up to 1 month. VSMCs were treated with the indicated amount of NAC for 8 hours before stimulation.

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Attenuated *Shigella* as a DNA Delivery Vehicle for DNA-Mediated Immunization

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Direct inoculation of DNA, in the form of purified bacterial plasmids that are unable to replicate in mammalian cells but are able to direct cell synthesis of foreign proteins, is being explored as an approach to vaccine development. Here, a highly attenuated *Shigella* vector invaded mammalian cells and delivered such plasmids into the cytoplasm of cells, and subsequent production of functional foreign protein was measured. Because this *Shigella* vector was designed to deliver DNA to colonic mucosa, the method is a potential basis for oral and other mucosal DNA immunization and gene therapy strategies.

Direct DNA-mediated immunization is an exciting new approach to vaccine development (*1*). We chose to exploit the ability of *Shigellae* to enter epithelial cells and escape the phagocytic vacuole as a method for directing plasmid DNA to the cytoplasm of the host cell for protein synthesis and processing for antigen presentation (*2*). To attenuate the *Shigella* vector, we made a deletion mutation in the *asd* gene encoding aspartate β-semialdehyde dehydrogenase, an essential enzyme that is required to synthesize the bacterial cell wall constituent

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diaminopimelic acid (DAP) (3, 4). The resultant 15D construct, a Δ*asd* isolate of *Shigella flexneri* 2a strain 2457T, was able to maintain the eukaryotic expression vector pCMVβ (5) in the absence of antibiotic-selective pressure. The plasmid pCMVβ expresses *Escherichia coli* β-galactosidase under the control of the immediate early promoter and enhancer from the human cytomegalovirus (CMV) in mammalian cells; this permitted easy analysis of mammalian cell–mediated gene expression after delivery.

Strain 15D was screened to ensure that the large plasmid that is essential for bacterial invasion of mammalian cells had not been lost during the genetic manipulations. Immunoblots verified that the strain continued to express the invasion-associated IpaB and IpaC polypeptides (6) and thus showed no loss of the invasion plasmid. To confirm earlier observations, we tested 15D and 15D(pCMVβ) for the ability to invade cultured baby hamster kidney (BHK) cells with and without DAP supplementation during the 90 min allowed for invasion (7–9). Examination by light microscopy of fixed and stained chamber slides revealed that in the absence of DAP, 15D and 15D(pCMVβ) entered 13% and 10% of the cultured BHK cells, respectively. By contrast, 33% (15D) and 29% [15D(pCMVβ)] of the BHK cells contained bacteria when DAP was present during the invasion step. Although both constructs were able to invade BHK cells, the addition of DAP during the invasion step increased the number of BHK cells infected and the number of viable bacteria recovered (9).

To test the ability of 15D to deliver plasmid DNA, we followed intracellular bacterial viability and β-galactosidase activity (Fig. 1) over a 48-hour time course (8, 10). Initially, 1×10^7 to 3×10^7 viable bacteria of each strain were recovered from monolayers of BHK cells with no detectable β-galactosidase activity in cell extracts. No β-galactosidase activity could be detected in bacterial extracts that were equivalent to the total number of bacteria added. At each assay point, a loss of 1 to 1.5 log units of viable bacteria occurred with no notable difference between strains 15D and 15D(pCMVβ). However, at both the 24- and 48-hour assay points, increasing units of β-galactosidase activity were readily detected in extracts of BHK cells infected with 15D(pCMVβ). The detected β-galactosidase activity did not result from expression within the bacteria because, although no activity was measured at the first two assay points, large numbers of viable bacteria were present. In addition, an isolate of 15D(pCMVβ) that did not express IpaB and IpaC (as measured by immunoblotting) was unable to bring about β-galactosidase activity at the 24-hour assay point.

Infected monolayers of BHK cells were immunostained to examine β-galactosidase expression within individual cells (Fig. 2) (8, 11). No intracellular immunostaining was observed in monolayers infected with

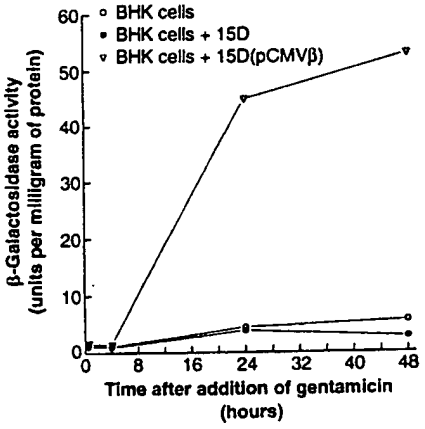

Fig. 1. Strain 15D was used as a carrier to deliver pCMVβ, a mammalian DNA expression plasmid, to BHK cells. β-Galactosidase activity (in units per milligram of protein) was determined for BHK cells alone (○), BHK cells infected with 3×10^9 15D (●), and BHK cells infected with 1×10^9 15D(pCMVβ) (▽). Determinations of β-galactosidase activity were made on an estimated 0.5×10^7 cells. Bacteria were grown as described (8). In this assay, DAP (50 μg/ml) was added to concentrated bacterial suspensions before these suspensions were added to flasks of semiconfluent BHK cells (~1×10^7 cells). At the indicated times, BHK cells were removed by trypsinization and washed in phosphate-buffered saline. A portion of the cell suspension was lysed with a 0.2% Triton X-100 solution, diluted, and plated on TSA Congo red DAP plates to determine the number of viable bacteria. β-Galactosidase activity was measured in the remaining cell extract by a standard biochemical assay (10) [units of β-galactosidase = 380 × OD_{420}/time (in minutes)]. β-Galactosidase activities were standardized to 1 mg of total protein, as determined with a BCA* protein assay kit (Pierce).

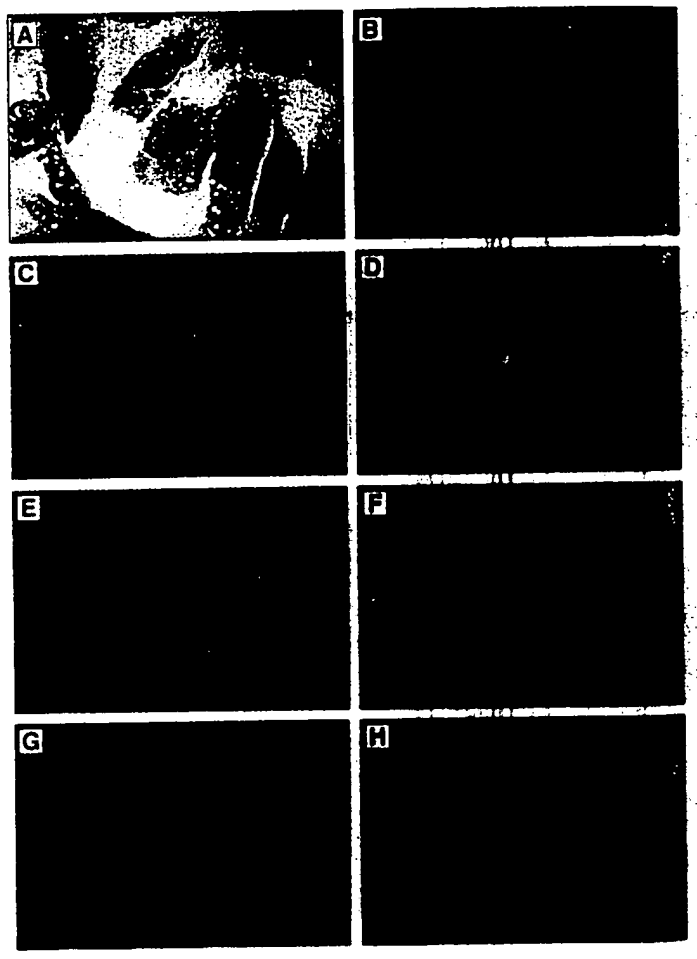

Fig. 2. Intracellular immunostaining to detect expression of β-galactosidase within BHK cells infected with 15D or 15D(pCMVβ). (**A**) A Leukostat-stained BHK monolayer infected with 15D(pCMVβ) 30 min after the addition of gentamicin-containing medium. (**B** through **H**) Immunostained infected BHK cells after the addition of gentamicin-containing medium. (B) 15D(pCMVβ), 30 min; (C) 15D, 4 hours; (D) 15D(pCMVβ), 4 hours; (E) 15D(pCMVβ), 24 hours; (F) 15D(pCMVβ), 48 hours; (G) 15D, 48 hours; (H) BHK cells alone. Three wells of a four-well chamber slide of BHK cell monolayers infected with 15D or 15D(pCMVβ) were immunostained to detect β-galactosidase expression (8, 10, 11). At the indicated times, washed monolayers were fixed in phosphate-buffered 4% paraformaldehyde for 5 min and then blocked with 3% goat serum (Gibco-BRL) in HBSS for 30 min. BHK cells were then permeabilized for 1 min with HBSS containing 0.1% saponin (Sigma). A monoclonal antibody to β-galactosidase (Sigma) was diluted 1:2000 in HBSS containing 0.1% saponin and applied for 30 min at 37°C in a humidified chamber. Fluorescein isothiocyanate–conjugated secondary antibody to mouse IgG (Fc-specific, Sigma) was diluted 1:32 and applied for 30 min at room temperature. Between each step, chamber slides were washed extensively with HBSS containing 0.1% saponin. A final wash step of HBSS alone was used to close permeabilized cells. Fluorescent images were visualized with a Nikon Microphot with epifluorescence attachment or with an Olympus VAN04-S with fluorescence attachment. Original magnifications, ×312.5 (A); ×62.5 (B through H).

either strain at the 30-min assay point (Fig. 2B). Only slight intracellular immunostaining was detected at the 4-hour assay point in monolayers infected with 15D(pCMVβ) (Fig. 2, C and D). By the 24- and 48-hour assay points, positive immunostaining of several cells per field was observed in monolayers infected with 15D(pCMVβ) (Fig. 2, E and F). Staining throughout the cytoplasm suggested that the plasmid DNA had been released from the bacterium into the cell cytoplasm, leading to transcription and translation by the mammalian cell. Immunostained cells also appeared to be rounded, possibly because of the presence of a large quantity of β-galactosidase protein. As measured by fluorescence-activated cell sorter (FACS) analysis, 1 to 2% of 5000 15D(pCMVβ)-infected BHK cells expressed β-galactosidase at the 24-hour assay point (*8*, *10*).

Visual examination of Leukostat-stained chamber slides of 15D(pCMVβ)-infected BHK cells indicated that 28% of the cells contained one to five visually intact bacterial cells, with 1.7% containing five bacteria (Table 1). Four hours after gentamicin treatment, 26% of the cells contained visually intact bacteria, with <1% of the cells containing four bacteria. Therefore, invasion with one to five bacteria was required for foreign gene expression. Because pCMVβ is a 7164–base pair plasmid that occurs in ~500 copies per bacterial cell, each bacterium is estimated to contain $\sim 3.93 \times 10^{-9}$ μg of DNA. Thus, intracytoplasmic delivery of no more than 4×10^{-9} to 20×10^{-9} μg of DNA by *Shigella* was sufficient for expression of β-galactosidase.

To demonstrate that gene delivery was not restricted to BHK cells, we infected murine P815 cells that express H-2^d class I major histocompatibility complex (MHC) molecules with 15D(pCMVβ). As shown in Table 2, 56.25 units of β-galactosidase activity were detected in lysates from P815 cells infected with 15D(pCMVβ). Further experiments will be necessary to determine whether these cells can present *Shigella*-delivered DNA-encoded foreign antigens in the context of class I.

Studies of the ability of 15D to deliver plasmid DNA in vivo have begun in two small animal models, the guinea pig keratoconjunctival and murine intranasal models, which are used to study *Shigella* pathogenicity and immunobiology (*12*, *13*). To determine whether 15D could deliver pCMVβ to the ocular surface of the guinea pig eye, we stained corneas for β-galactosidase activity and visually examined them at various times after inoculation (*12*). Varying amounts of staining were observed in the outer region of the cornea near the sclera of the right eyes that received 15D(pCMVβ), except those from day 8, in which staining was detected in only one of three corneas. Several areas typical of the staining observed in corneas that received 15D(pCMVβ) are shown in Fig. 3B. No apparent endogenous β-galactosidase activity was detected in eyes inoculated with 15D. Histology experiments will be needed to examine in greater detail the percentage of cells and cell type(s) invaded by 15D(pCMVβ) and those staining positive for β-galactosidase. In an initial experiment, spleen cells from intranasally inoculated BALB/c mice showed a moderate proliferative response to β-galactosidase protein (2.5 μg/ml) (*13*, *14*). The stimulation index (*14*) was 3.6 when the inoculum was supplemented with DAP compared with 2.1 in the absence of DAP. Although preliminary, these experiments indicate that bacteria can be used to deliver plasmid DNA in vivo.

Our method for delivering functional DNA inside cells need not be restricted to *Shigella* because the invasion genes used by *Shigella* can be inserted into other bacteria such as *E. coli* (*15*). Likewise, other bacteria such as *Listeria* are able to invade cells and break out of the phagocytic vacuole into the cytoplasm (*16*). Although we have no formal proof that such a release of bacteria from the phagocytic vacuole into the cell cytoplasm is essential for DNA delivery, preliminary experiments with *Salmonella typhimurium*, which reaches the cytoplasm only with difficulty, suggest that this organism is not an efficient DNA delivery vehicle (*17*).

Any bacterial DNA delivery system will need to strike a balance between cell inva-

Table 1. Percentage of BHK cells infected and number of bacteria per BHK cell, as shown by microscopic examination. Chamber slides and bacteria were prepared as described (*8*). At least 400 BHK cells of each group were examined.

Elapsed time (hours)	BHK cells infected (%) (mean)	Bacteria per infected BHK cell (mean ± SD)	Number of BHK cells containing 1 to 6 bacteria						
			1	2	3	4	5	6	Total
			Strain 15D						
0.5	39	1.84 ± 1.2	96	47	14	14	3	3	177
4	36	1.68 ± 0.94	106	36	13	5	0	1	161
24	3.7	1	17	–	–	–	–	–	17
48	2.2	1	10	–	–	–	–	–	10
			Strain 15D(pCMVβ)						
0.5	28	1.35 ± 0.72	76	29	7	5	2	0	119
4	26	1.40 ± 0.74	95	16	4	1	0	0	116
24	3.3	1	14	1	–	–	–	–	15
48	3.8	1	18	–	1	–	–	–	19

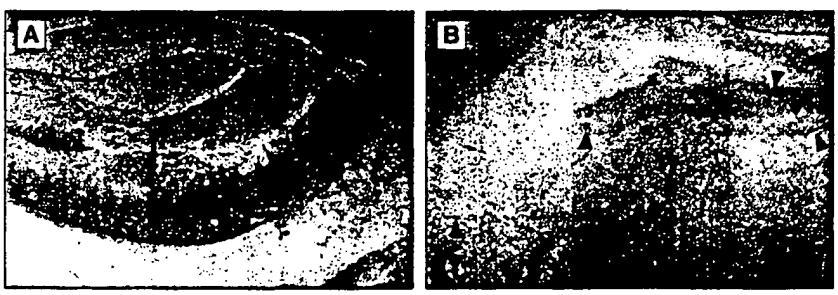

Fig. 3. Ability of 15D to deliver pCMVβ to ocular tissue. (**A**) Left cornea (15D) and (**B**) right cornea [15D(pCMVβ)], 48 hours after ocular inoculation. Arrowheads indicate areas of β-galactosidase staining.

Table 2. β-Galactosidase activity in P815 cells after infection with 15D(pCMVβ). Bacteria used to infect P815 cells were grown as described (*8*). After addition of the bacterial cultures containing DAP to the nonadherent P815 cells cultured in six-well plates, the plate was spun at 500*g* for 5 min. Bacteria and P815 cells were allowed to interact for 90 min. The cells were then extensively washed with DMEM and resuspended in DMEM containing gentamicin (100 μg/ml) for a 1-hour incubation at 37°C in the presence of 5% CO_2. The cells were again extensively washed and resuspended in DMEM containing gentamicin (20 μg/ml) for overnight culture at 37°C in the presence of 5% CO_2. β-Galactosidase activity and protein concentrations were determined at 24 hours as described (*8*, *10*).

Source	β-Galactosidase (units per milligram of protein)
P815 cells	3.04
P815 cells + 15D	5.62
P815 cells + 15D(pCMVβ)	56.25

sion (with its subsequent reactogenicity) and efficiency of delivery. In the case of *Shigella*, the genes responsible for invasion also cause invasion and apoptosis of macrophages, followed by inflammation (*18*). We constructed a *Shigella* strain that was completely unable to divide in the absence of DAP. Determination of the safety of this strain awaits human trials. Preliminary experiments with a guinea pig keratoconjunctivitis challenge model indicate that a two-dose immunization regimen followed by a challenge with virulent *Shigella* 3 weeks later gave 100% protection (*12*, *14*). These results demonstrate that this highly attenuated strain, which is capable of DNA delivery, functions well in vivo.

The bacterial DNA delivery system described here has several advantages for certain applications. Delivery of DNA-encoded antigens to the mucosal immune system should permit mucosal immunization simultaneously with multiple antigens that (i) can be directed for class I presentation, class II presentation, or both; (ii) can stimulate T helper cells (T_H1 or T_H2); and (iii) can be secreted while maintaining the proper folding and conformational epitopes for immunoglobulin A (IgA) and immunoglobulin G (IgG) antibody production. Diseases that may be especially responsive to this approach include diarrheal diseases such as rotavirus, gastrointestinal diseases such as the ulcer-causing *Helicobacter pylori*, and sexually transmitted disease agents such as human immunodeficiency virus, *Neisseria gonorrhoeae*, and human papilloma virus. Suppression of autoimmunity through manipulation of immune tolerance mechanisms in the gut has been demonstrated (*19*), and if such a technique proves to be generally applicable, it should also be amenable to our approach.

Perhaps the greatest advantage of bacterial delivery of DNA for vaccination and for potential gene therapy and replacement is the ease and acceptability of oral and other forms of mucosal delivery. Likewise, because no DNA purification is required for this type of DNA vaccination (which is essentially a live, attenuated bacterial vector), vaccines can be produced for the cost of fermentation, lyophilization, and packaging. Therefore, this type of vaccination may represent, at least in part, a solution to the costs and difficulties inherent in the production and development of current vaccines.

Aside from the practical applications of bacterial DNA delivery, the relatively efficient ability of *Shigella* to transfer functional DNA containing a eukaryotic promoter into mammalian cells leads to speculation concerning the potential role of such a mechanism in evolution. Plasmids for vaccine use are designed so as to minimize the possibility of chromosomal integration, but in nature this may not be the case.

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4. A *Δasd* derivative of *Shigella flexneri* 2a strain 2457T was constructed by polymerase chain reaction (PCR) amplification of an *E. coli asd* gene [C. Haziza, P. Stragier, J. C. Patte, *EMBO J.* **1**, 379 (1982)] incorporating Bgl II restriction sites (forward primer, 5′-Bgl II-CCCTGATAATTGCCGC-3′; reverse primer, 5′-Bgl II-CGCTTACTCCTGTATTACGC-3′) for directed cloning into a previously described vector (A. Branstrom, D. Sizemore, R. Warren, J. Sadoff, paper presented at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, 20 October 1993). Positive clones were selected with the use of *E. coli* χ6097 (*3*). Reverse PCR amplification was used to delete 553 base pairs of the *E. coli asd* structural gene (positions 439 to 991) (forward primer, 5′-GATCCTCAACACATCTTCCG-3′; reverse primer, 5′-CGAGGGCCTTTAGCGCCTCC-3′). The kanamycin resistance cassette from pUC4K-KIXX (Pharmacia) was digested with Sma I and cloned between the flanking *asd* sequences. A 2-kb fragment of the remaining *asd* gene with the internal kanamycin resistance cassette was PCR-amplified (forward primer, 5′-Sac I-CCCTGATAATTGCCGC-3′; reverse primer, 5′-Sal I-CGCTTACTCCTGTATTACGC-3′) and cloned into the Sac I–Sal I site of the positive selection suicide vector pCVD442 [M. S. Donnenberg and J. B. Kaper, *Infect. Immun.* **59**, 4310 (1991)], creating pSEAK. Clones were transformed into SM10λpir [R. Simon, U. Prieter, A. Puhler, *Biotechnology* **1**, 784 (1983)] and selected for resistance to ampicillin. SM10λpir(pSEAK) was conjugated with *S. flexneri* 2a strain 2457T (pAB322[Tet^r,Amp^s]) and conjugants were selected for resistance to ampicillin and tetracycline. PCR analysis of resultant isolates showed that plasmids integrating into the chromosome had recombined with the downstream portion of the *asd* fragment of pSEAK. Growth on sucrose resulted in a second recombination event [J. Quandt and M. F. Hynes, *Gene* **127**, 15 (1993)], and isolate 15C was obtained by screening the resultant isolates for resistance to kanamycin and the requirement for DAP. Hybridization and PCR analysis confirmed a deletion in *asd*. Further, the mutation could be complemented with a low–copy number plasmid carrying the *E. coli asd* gene. Isolate 15C was cured of the tetracycline resistance plasmid by fusaric acid treatment [S. R. Maloy and W. D. Nunn, *J. Bacteriol.* **145**, 1110 (1981)] to generate isolate 15D.

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8. The general assay used for these studies was adapted from those described previously for *Shigella* plaque analysis (*6*, *7*). A single Congo red–binding positive colony (denoting the expression of plasmid-encoded *Shigella* virulence determinants) of each strain was used to inoculate overnight LB broth cultures containing DAP (50 μg/ml) (for 15D) or DAP plus ampicillin (250 μg/ml) [for 15D(pCMVβ)]. Overnight cultures were diluted 1:50 and grown to approximately mid–log phase in the presence of DAP. For chamber slide assays (Nunc) and 24-well plate assays, 200 μl of washed bacteria in Hanks' balanced solution (HBSS) with or without DAP (50 μg/ml) was added to three wells of semiconfluent BHK cells (1×10^5) at ~100:1. For flask assays, bacteria were grown as described, except that mid–log phase cultures were concentrated 10-fold and 2 ml of the bacterial solution was added to a flask of BHK cells (~1×10^7). In all assays, bacteria were allowed to interact with the BHK cells in this minimal volume for 90 min at 37°C in the presence of 5% CO_2. Nonadherent bacteria were removed by extensive washes with HBSS. Extracellular bacteria were then killed by the addition of Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (BioWhittaker) and gentamicin (50 μg/ml). For plating assays, cells were lysed with a 0.2% Triton X-100 solution and appropriate dilutions were plated on tryptic soy agar (TSA) Congo red DAP plates for determination of viable bacterial counts. At the indicated times, the chamber slides were extensively washed, fixed, and immunostained or stained with a Leukostat set (Fisher) before light or fluorescent microscopy. An Instat statistical program (Graphpad, San Diego, CA) was used to calculate means, SDs, and SEs. All data presented are representative of experiments performed two or more times.
9. The ability of a *Δasd* derivative of *S. flexneri* 2a strain 2457T to invade mammalian cells in culture was assayed to examine the requirement of DAP during the adherence and invasion step. Bacterial solutions with or without DAP were allowed to interact with BHK cells for 90 min, washed extensively, and then treated with gentamicin-containing media for 30 min before plating. In the absence of DAP, 1070 ± 404 (15D) and 1095 ± 332 [15D(pCMVβ)] viable bacteria were recovered, versus $8.2 \times 10^4 \pm 1 \times 10^4$ (15D) and $8.6 \times 10^4 \pm 3.5 \times 10^4$ [15D(pCMVβ)] when DAP was present [mean ± SE; $P = 0.024$ (Mann-Whitney test)].

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12. For guinea pig inoculations, 7.2×10^8 15D bacteria in a 25-μl volume were placed on the left eye and 4×10^8 15D(pCMVβ) bacteria in a 25-μl volume were placed on the right eye, as described [A. B. Hartman, C. J. Powell, C. L. Schultz, E. V. Oaks, K. H. Eckels, *Infect. Immun.* **59**, 4075 (1991)]. On days 1, 2, 6, and 8, animals were killed and each eye was removed for dissection of corneas and adjacent sclera. Whole eyes or dissected corneas were stained for β-galactosidase [J. R. Sanes, J. L. R. Rubenstein, J. Nicolas, *EMBO J.* **5**, 3133 (1986)]. After overnight development, corneas were examined under a dissecting microscope, scored for staining, and photographed. Corneas from three guinea pigs were examined on days 1, 2, and 8; corneas from two guinea pigs were examined on day 6. All animal experiments were conducted in adherence to principles stated in the *Guide for Care and Use of Laboratory Animals* (NIH publication 86-23, National Institutes of Health, Bethesda, MD, 1985).

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